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ORIGINAL PAPER

Guang-Huai Jiang · Zhi-Hui Xia · Yong-Li Zhou
Jia Wan · Da-Yong Li · Run-Sheng Chen
Wen-Xue Zhai · Li-Huang Zhu

Testifying the rice bacterial blight resistance gene *xa5* by genetic complementation and further analyzing *xa5* (*Xa5*) in comparison with its homolog *TFIIA*γ*1*

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Abstract The recessive gene *xa5* for resistance to bacterial blight resistance of rice is located on chromosome 5, and evidence based on genetic recombination has been shown to encode a small subunit of the basal transcription factor IIA (Iyer and McCouch in MPMI 17(12):1348–1354, 2004). However, *xa5* has not been demonstrated by a complementation test. In this study, we introduced the dominant allele *Xa5* into a homozygous *xa5*-line, which was developed from a cross between IRBB5 (an *indica* variety with *xa5*) and Nipponbare (a *japonica* variety with *Xa5*). Transformation of *Xa5* and subsequent segregation analysis confirmed that *xa5* is a V39E substitution variant of the gene for TFIIAγ on chromosome 5 (*TFIIA*γ5 or *Xa5*). The rice has an addition gene for TFIIAγ exists on chromosome 1 (*TFIIA*γ1). Analysis of the expression patterns of *Xa5* (*TFIIA*γ5)/*xa5* and *TFIIA*γ1 revealed that both the genes are constitutively expressed in different rice organs. However, no expression of *TFIIA*γ1 could be detected in the panicle by reverse transcriptase-polymerase chain reaction. To compare the structural difference between the Xa5/xa5 and TFIIAγ1 proteins, 3-D structures were predicted using computer-aided modeling techniques. The modeled structures of Xa5 (xa5) and TFIIAγ1 fit well with the structure of TFIIA small subunit from human, suggesting that they may all act as a small subunit of TFIIA. The E39V substitution in the xa5 protein occurs in the α-helix domain, a supposed conservative substitutable site, which should not affect the basal transcription function of TFIIAγ. The structural analysis indicates that *xa5* and *Xa5* potentially retain their basic transcription factor function, which, in turn, may mediate the novel pathway for bacterial blight resistance and susceptibility, respectively.

Keywords Bacterial blight resistance · Recessive resistance gene · Basal transcription factor IIA · Gene duplication rice (*Oryza sativa* L.)

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Guang-Huai Jiang and Zhi-Hui Xia contributed equally to this work

G.-H. Jiang · Z.-H. Xia · D.-Y. Li · W.-X. Zhai (✉)
L.-H. Zhu (✉)
Institute of Genetics and Developmental Biology,
Chinese Academy of Sciences, 100101 Beijing, China
E-mail: lhzhu@genetics.ac.cn
Tel.: +86-10-64870491
Fax: +86-10-64873428

Y.-L. Zhou
Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, 100081 Beijing, China

J. Wan · R.-S. Chen
Institute of Biophysics, Chinese Academy of Sciences, 100101 Beijing, China

Introduction

Plants are vulnerable to the infections of all kinds of pathogens including bacteria, fungi, and viruses. However, plants have also evolved diverse resistance mechanisms to effectively react and survive from such infections. Some important resistance reactions in plants can be concisely described by the "gene-for-gene" hypothesis. Namely, the resistance of a plant to a pathogen infection can only be formed if the plant has a specific resistance gene (R-gene) against a specific corresponding pathogen avirulence (avr) gene (Flor 1955; Staskawicz et al. 1995). The hypothesis has been directly tested upon the cloning of numerous R and avr genes from resistant plants and incompatible pathogens. To date, more than 40 plant disease R-genes have been cloned from several plant species (Martin et al. 2003). Most plant R-genes are dominant, while a few, including *mlo*, *pvr-2*, and *RRS1-R*, are recessive. The recessive *mlo* gene in barley encodes a membrane-anchored protein with seven transmembrane domains and mediates a

broad, non-race-specific resistance reaction to the powdery mildew fungus, *Erysiphe graminis* f sp. *Hordei* (Büschages et al. 1997). The recessive gene *pvr2* in pepper encodes an eukaryotic initiation factor 4E that confers resistance against the strains of potato virus Y (Ruffel et al. 2002), and the *Arabidopsis* recessive gene *RRS1-R* encodes a protein containing Toll-IL-1-NBS-LRR with a WRKY domain and confers resistance against several strains of *Ralstonia solanacearum* (Deslandes et al. 2002). Obviously, the studies on the recessive genes with plant disease resistance are currently rather limited as compared with those on dominant genes.

Rice bacterial blight, caused by *Xanthomonas oryzae pv. oryzae* (*Xoo*), is one of the most serious bacterial diseases in rice growing area worldwide. By now, more than 26 bacterial blight resistance genes have been identified, from which four dominant *Xa21*, *Xa1*, *Xa26*, and *Xa27* have been cloned (Song et al. 1995; Yoshimura et al. 1998; Sun et al. 2004; Gu et al. 2005). *xa5* is an important race-specific recessive gene in rice breeding due to its broad resistance spectrum to most *Xoo* strains. Pyramid lines with *xa5* gene and other R-genes have a higher and wider spectrum of resistance than each of the single gene lines (Huang et al. 1997). This indicates that the resistance pathway governed by *xa5* is different from other genes and may interact with other pathways by the dominant genes such as *Xa21* and *Xa24*. Therefore, cloning of *xa5* gene will shed light on its unique resistance mechanism to *Xoo*. In our previous study (Zhong et al. 2003), we mapped *xa5* to a 24-kb interval including two predicted genes on rice chromosome 5 and identified the one that encodes the small (γ) subunit of the basal transcription factor (TFIIAγ) as the most possible candidate gene for *xa5*. Iyer and McCouch (2004) then identified *xa5* as a gene for the general eukaryotic transcription factor by their fine mapping. In the study, they found seven nucleotide substitutions, including two in the first open reading frame (ORF1) and five in 3′ untranslated region (3′UTR) between the resistant (IRBB5) and susceptible (IR24) lines. The two substitutions in ORF1 results in an amino acid change from glutamic acid in the resistant line to valine (E39V) in the susceptible line in the N-terminus of the xa5 candidate protein. By sequencing the region around the substitutions in 27 resistant and nine susceptible accessions from the group of Aus-Bori rice varieties, they further demonstrated the relationship of the changed amino acid at position 39 to the resistance phenotype. However, they did not complement the recessive allele with the dominant gene *Xa5*.

Interestingly, TFIIA is one of a set of general transcription factor required by RNA polymerase II, which has been identified as a two-subunit complex in yeast and as a three-subunit complex in higher eukaryotes (DeJong and Roeder 1993; Ranish and Hahn 1991). As a basal transcription factor in eukaryotes to initiate the mRNA synthesis, it can be assembled into a transcriptionally competent pre-initiation complex with TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (Orphanides et al. 1996). The predicted amino acid sequences of TFIIA across yeast, Drosophila and man show a remarkable conservation, and especially their small subunits (TFIIAγ) are highly homologous (Li et al. 1999). Like Drosophila, rice has two *TFIIA*γ-like genes in its genome, one corresponding to *xa5* on chromosome 5 and the another, *TFIIA*γ1, on chromosome 1 (http://www.rgp.dna.affrc.go.jp/IRGSP/), in contrast to *Arabidopsis*, which has only one of the genes (http://www.arabidopsis.org). Obviously, redundancy of *TFIIA*γ may facilitate the emergence of a new resistance gene and raises two questions. Are the two *TFIIA*γ-like genes still highly conserved in their functional domains, and how different the xa5 protein from either of Xa5 or TFIIAγ1?

Here we report our independent study on the *xa5* gene with the results of the complement test. We also present predictive models of the 3-D structures of the Xa5, xa5, and TFIIAγ1 proteins by using computer-aided modeling techniques. The implications of the structural modeling are also discussed.

Materials and methods

Plant material

Nipponbare (*japonica*) was previously tested and shown to contain the dominant allelic site of *xa5* gene (data not shown). IRBB5 (*indica*) is a near isogenic line of IR24 containing homozygous *xa5* gene (Yoshimura et al. 1992). An F_2 population with 20,000 plants was generated from a cross of Nipponbare and IRBB5 for fine mapping *xa5* and to move the xa5 gene into rice with a high transformation frequency. Resistant F_2 plants were used to self-fertilize to produce the F_3 progeny. The F_3 plants were checked with the close flanking marker of *xa5* gene (SSR3, SSR12, and K8, and S5/NcoI; Fig. 1a) to ensure that they have *xa5* gene from IRBB5, and one of them referred to as NBB5 was used as the recipient rice for transformation in this study (Fig. 2 and Tables 1, 2).

Bacterial inoculation and evaluation

Six Philippine *Xoo* races (race 1, POX 61; race 2, PXO86; race3, PXO79; race 4, PXO71; race 5, PXO112; race 6, PXO99; Mew 1987) were used in this study. When the rice grows to tillering stage, three to five uppermost fully expanded leaves of them were inoculated using the leaf-clipping method (Kauffman et al. 1973). The phenotype scoring was carried out when the development of the disease spots in the susceptible plants was stable after 15 days (Zhong et al. 2003).

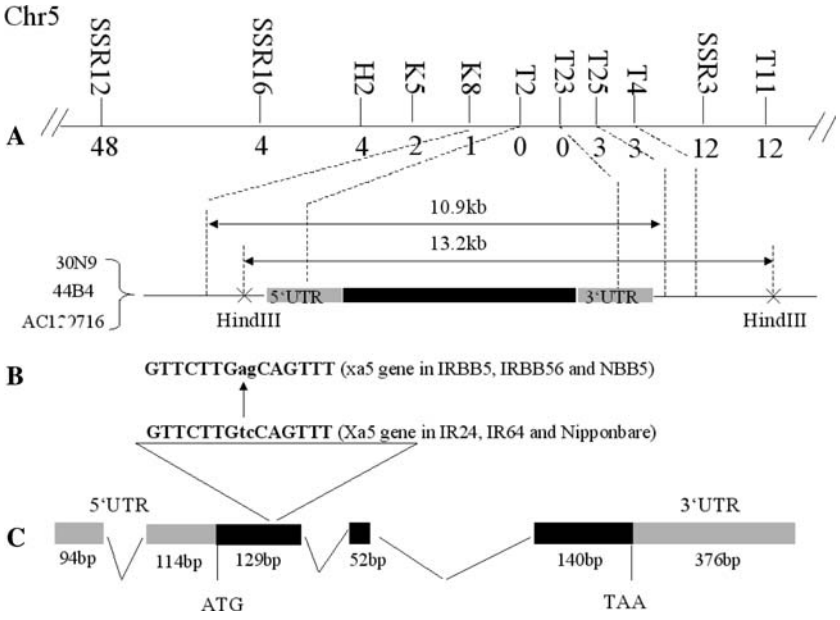

Fig. 1 Mapping of *xa5* gene and the analysis of its structure. **a** A fine genetic map of the *xa5* region. *xa5* was mapped between CAPS marker K8 and T4, and co-segregated with T2 and T23 marker on chromosome 5. The proximal flanking markers K8 and T25 were separated from *xa5* by one and three recombination events, respectively. The numbers under the line represent recombinant individuals of a given marker in the population of 20,000 plants. **b** The 13.2 kb genomic region containing *Xa5* in the BAC clone AC129716, AC079022, and 44B4. **c** Structure of the *xa5* gene. The start and stop codons are indicated. Exons are indicated by *red squares*, and introns by *lines* angled downward. The di-nucleotide substitution is shown in the second exon

Table 1 The PCR-based molecular markers developed in this study

Marker	Forward primer	Reverse primer	Size (bp)	Restriction enzyme or repeats
K8	ctacagagaactaatcaacc	ctcgatttcttacaatgtcc	704	Hinf I
T23	gtatcaatcagtcctctggg	aggggtgtagtgttatggg	831	Rsa I
T25	tccagtcgaaaggtcttcac	ttgcagactcgcagttactc	837	Alu I
D5	gctcgccattcaagttctcg	tgctcttgacttggttctcc	165	XhoI
S5	ctctctacttttgtctgg	ccaaacacagatgagcag	1,024	NcoI
SSR3	cgagatcgagatcgagatgc	cgaaaggatgggaggagaagc	182	$(ctt)_{13}$
SSR12	ctgaaagcattatccctccc	cgtttcggtttctctctccc	183	$(at)_{31}$

Xa5 cloning

The IR64 BAC library containing the dominant *Xa5* (Yang et al. 1998) was screened, and a positive clone 44B4 containing the *Xa5* gene was obtained in previous study (Zhong et al. 2003). To isolate the candidate gene region from the BAC clone, the sequence of AC129716 BAC clone from IRGSP were analyzed using the software package DNAstar (www.DNAStar.com). A 13.2 kb *Hin*dIII fragment was shown to contain the entire candidate gene with its native promoter. This fragment was subcloned into the *Hin*dIII-digested vector pCAMBIA1300 to generate pCAMBIA1300::Xa5 plasmid for the complementation test.

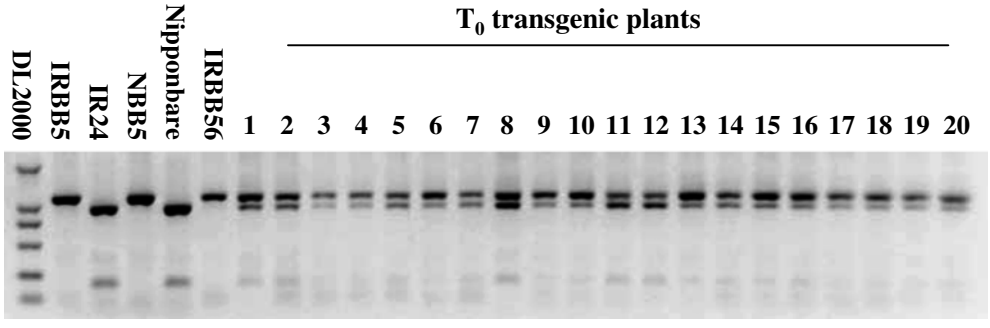

Fig. 2 PCR analysis of selected T_0 transgenic plants and their parents with the CAPS marker S5/NcoI. (20 T_0 transgenic plants were randomly selected from the 42 hygromycin-resistant transformed plants)

Table 2 Evaluation of bacterial blight resistance in T_0 transgenic plants with pCAMBIA1300::Xa5

SN	Evaluation of bacterial blight resistance		PCR check, S5/NcoI	SN	Evaluation of bacterial blight resistance		PCR check, S5/NcoI
	Mean lesion length/mean leaf length (cm)	Resistance/susceptible (R/S)			Mean lesion length/mean leaf length (cm)	Resistance/susceptible (R/S)	
1	9.4/19.1	S	z	23	11.8/22.2	S	z
2	8.4/21.3	S	z	24	10.7/22.0	S	z
3	10.9/30.5	S	z	25	11.0/27.2	S	z
4	3.8/12.7	R	y	26	14.7/35.8	S	z
5	6.1/18.2	S	z	27	11.2/38.0	S	z
6	1.5/7.0	R	y	28	11.5/26.2	S	z
7	12.0/24.5	S	z	29	8.8/21.0	S	z
8	14.5/33.7	S	z	30	10.3/33.0	S	z
9	11.9/23.7	S	z	31	1.8/30.7	R	y
10	6.3/22.4	S	z	32	15.8/38.3	S	z
11	8.7/26.4	S	z	33	11.8/28.0	S	z
12	11.5/16	S	z	34	8.7/32.8	S	z
13	11.2/22.5	S	z	35	14.8/44.2	S	z
14	13.5/23.8	S	z	36	16.8/32.5	S	z
15	8.8/21.0	S	z	37	8.1/22.2	S	z
16	11.2/27.2	S	z	38	13.5/18.5	S	z
17	20.8/24.0	S	z	39	9.1/29.8	S	z
18	11.8/21.5	S	z	40	6.9/22.3	S	z
19	12.8/27.5	S	z	41	1.1/14.0	R	y
20	8.8/17.5	S	z	42	6.9/28.7	S	z
21	11.8/28.2	S	z	43	4.9/17.0	S	x
22	11.2/23.2	S	z	44	1.9/21.9	R	y

The first 42 plants are T_0 transgenic plants, the 43rd plant is Nipponbare, and the 44th plant is NBB5 (The PCR band type of Nipponbare and NBB5 plants were indicated by *x* and *y*, respectively; the band type containing both bands of Nipponbare and NBB5 was indicated by *z*)

Transformation

The binary plasmid pCAMBIA1300::Xa5 and the empty plasmid pCAMBIA1300 were introduced into *Agrobacterium tumefaciens* LBA4404 by electroporation, and subsequently transformed to NBB5 plants as described (Zhai et al. 2001).

Bacterial growth curve assay

The growth curve analyses were performed as described (Song et al. 1995). The bacterial populations were determined from two to three leaf samples at each time point. Leaf tips were ground in sterilized water and plated on PSA medium (10 g/l tryptotone peptone, 10 g/l sucrose, 1 g/l glutamic acid, 16 g/l agar, pH 7.0) with 100 mg/ml cycloheximide and 200 μM 5-Azacytidine.

Reverse transcriptase-polymerase chain reaction

To determine the expression of the candidate gene, 1-month-old Nipponbare and NBB5 plants were inoculated with Philippine race 1 (PXO61) of Xoo and water. Two grams of leaves were harvested for total RNA isolation at 12, 24, 48, and 96 h after inoculation (Sambrook et al. 1989). The first-strand cDNA was reverse-transcribed from the DNaseI treated total RNA with oligo (dT) primer and amplified with a start PCR cycle of 2 min 30 s at 94°C, 1 min at 55°C, 2 min at 72°C, then followed by 25 or 30 cycles (45 s at 94°C, 1 min at 55°C, 1 min at 72°C) and 5 min at 72°C. The primers for the Xa5/*xa5* gene reverse transcriptase-polymerase chain reaction (RT-PCR) were 5′-agtttaggcgcacgcatcg-3′(sense) and 5′-aggaaggggagggtgact-3′(antisense); primers for *TFIIAγ1* were 5′-tgacaagtccatgactagc-3′(sense) and 5′-ctcttctttagtctccagc-3′(antisense); primers for hygromycin were 5′-gcgagtacttctacacagcc-3′ (sense) and 5′-ctgcctgaaaccgaactgcc-3′ (antisense) and those for actin were ActF and ActR (5′-tatggtcaaggctgggttcg-3′ and 5′-ccatgctcgatggggtactt-3′).

Phylogenetic analyses

The neighbor-joining tree (Saitou and Nei 1987) of Xa5, xa5, TFIIAγ1, and other TFIIAγ protein sequences were constructed by using the program of PHYLIP version 3.572c (Felsenstein 1985). Bootstrap analysis (Felsenstein 1997) was carried out with 1,000 replicates. The phylogenetic trees were constructed with Kimura two-parameter distances (Kimura 1980). TreeView was used to generate the graphical output (Page 1996). The numbers at the branching point indicate the percentage of times that each branches topology was found during bootstrap analysis ($n = 1{,}000$).

Prediction of 3-D structure of Xa5, xa5, and TFIIAγ1

Initial structure of Xa5, xa5, and TFIIAγ1 were modeled by knowledge-based homology modeling using the software package Insight II, Discover 3, Homology of MSI, Inc. on SGI graphic workstation (Chen et al. 1996). The starting scaffold for homology modeling was the X-ray crystallographically determined structure of human TFIIA D chain (PDB ID: 1NVP). These crude initial structures were optimized by molecule dynamics simulation with the software package Gromacs 3.1.4 (Department of Biophysical Chemistry, University of Groningen). First, the B, C, and D chain of human TFIIA were energy minimized in vacuum, then solved in water box (PBC) and added relevant ions to energy minimize all the system. Second, fixed the position of chains B and C, then heated the system to 300°K in 600 ps; the time step is 1 fs. Third, the free dynamics simulations were carried out in 600 ps, and the time step is 3 fs. Fourth, equilibrate the system for 600 ps, then save a structure every 100 steps and draw a potential energy–time plot. At the end of the dynamics simulation, the conformation with lowest potential energy was picked for the next cycle of refinement. This combination of energy minimization and dynamics was repeated until satisfactory conformational parameters were obtained.

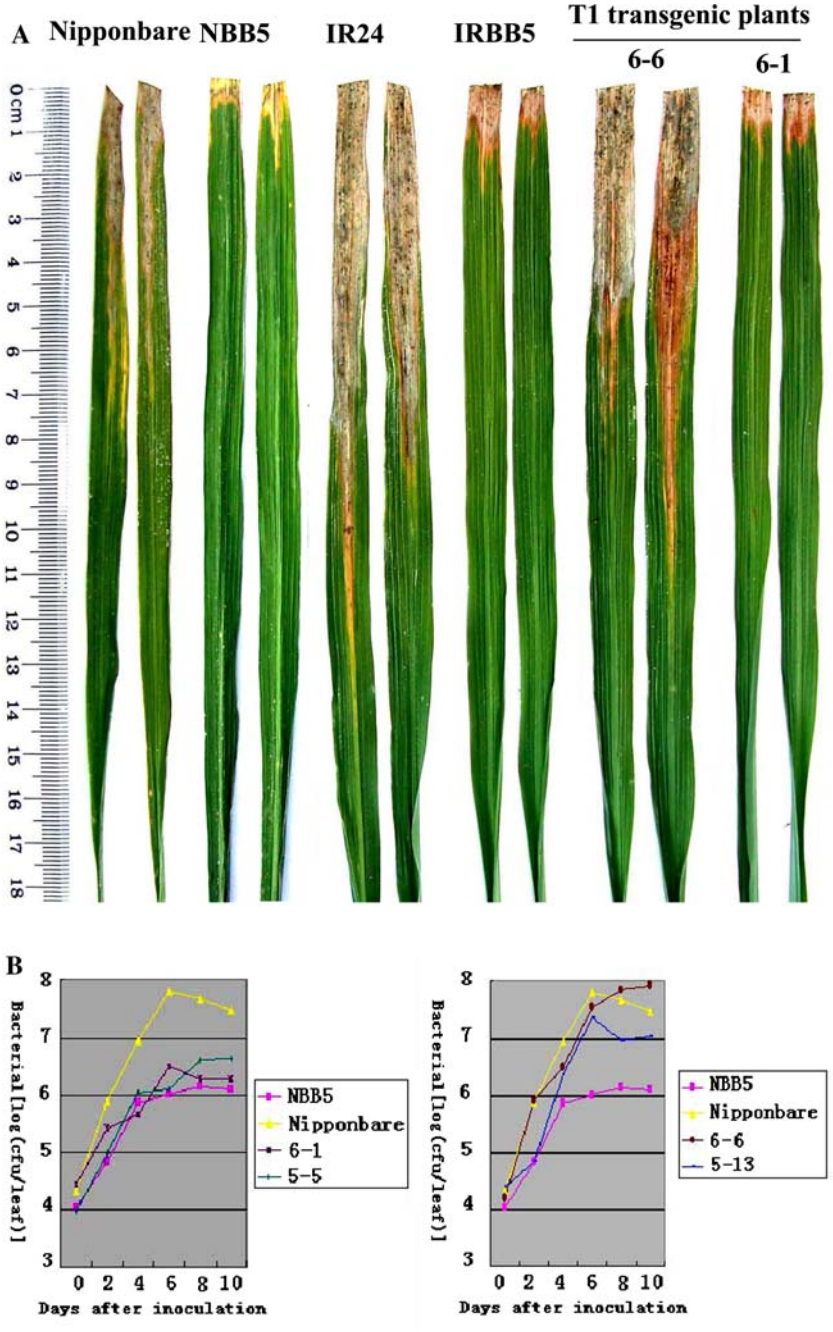

Fig. 3 Susceptible to Philippine race 1 conferred by the cloned *Xa5* gene. **a** The phenotypes of T_1 transgenic plants and their parents (*1* Nipponbare, *2* NBB5, *3* IR24, *4* IRBB5, *5* the susceptible T_1 transgenic plant, *6* the resistant T_1 transgenic plant). **b** Growth rates of *Xoo* strain Philippine race 1 on the leaves of T_1 transgenic rice containing homozygous *Xa5* gene (5–13 and 6–6) and *xa5* gene (5–5 and 6–1), NBB5 with homozygous *xa5* gene and the control Nipponbare. The bacterial populations of susceptible and resistant T1 plants were determined from two to three leaf samples at each time point

Results

Generating a recipient rice line for the transformation of *Xa5*

The map-based cloning approach in rice usually involves a complementation test to confirm the function of the candidate gene. However, some *indica* rice lines, and IRBB5 in particular, are difficult to be transformed. In this study, we crossed IRBB5 (*indica*) with a susceptible variety, Nipponbare (*japonica*), to generate resistant lines with the *xa5*/*xa5* genotype in a genetic background similar to Nipponbare for our transformation experiments. One of the resistant F_2 plants carrying *xa5* gene from the cross was selected through molecular marker analysis and field examination. Self-crossed F_3 plants containing *xa5* were verified with an inner CAPS (cleaved amplified polymorphic sequence) marker S5/NcoI for the *xa5* gene sequence (Fig. 1a) and its flanking markers (data not shown). One of these homozygous *xa5*-containing lines, referred to as NBB5, that satisfied all the requirements for transformation was chosen for the complementing test (Fig. 2 and Table 2).

Functional complementation for susceptibility of *xa5*-containing lines by *Xa5*

We have independently mapped *xa5* into a 10.9 kb fragment of a rice BAC clone AC129716 (http://www.rgp.dna.affrc.go.jp/IRGSP/) on chromosome 5 with an F_2 population containing 20,000 plants constructed from the cross between Nipponbare and IRBB5 (Fig. 1a; Zhong et al. 2003). We used the 10.9 kb sequence to blast against the library of 28,000 cDNA clones (http://www.cdn01.dna.affrc.go.jp/cDNA) and identified a clone, AK065182, corresponding to TFIIAγ. To clone the genomic gene for *Xa5*, we used the flanking markers K5 and T4 of the *xa5* gene (Fig. 1a) to screen the BAC library constructed from the rice variety IR64 that contains the dominant allelic gene *Xa5* by Yang et al. (1998). A positive clone 44B4 was obtained and confirmed to contain a 13.2 kb *Hin*dIII fragment. The only detectable ORF in this fragment was proved the dominant susceptible gene *Xa5* by sequencing (Fig. 1b). This fragment was then introduced into the NBB5 plants by *Agrobacterium*-mediated transformation. A total of 42 hygromycin-resistant transgenic plants were obtained and inoculated with Philippine race 1 (P1). Of these, 37 plants showed a susceptible phenotype. Moreover, these susceptible plants were all characterized as *Xa5*-containing transgenic plants by the *Xa5*-specific marker S5/NcoI (Fig. 2 and Table 2), whereas the transgenic plants with the empty plasmid pCAMBIA1300 remained resistant as NBB5.

To further confirm that the susceptible phenotype was due to the complementation of the transgene *Xa5*, the self-pollinated progenies (T_1 lines) from 27 independent susceptible T_0 plants were inoculated with P1. From each of the T_0 progenies, resistant and susceptible plants segregated in various ratios (Fig. 3a and detailed data not shown). The resistance spectrum of the segregated T_1 resistant plants was found the same as that of the gene-donor parent IRBB5 by inoculating six philippine *Xoo* races (data not shown), indicating that the race-specific resistance gene *xa5* in NBB5 was not changed by transformation, and that the dominant transgene *Xa5* complemented the function of the recessive resistance gene *xa5* in the transgenic plants.

We measured the growth rates of *Xoo* race 1 (PXO61) on resistant and susceptible T_1 plants. The bacterial growth rates on the susceptible plants were similar to that on the susceptible variety Nipponbare, which were 100-fold higher than that on the resistant variety, NBB5 (Fig. 3b). In contrast, the bacterial growth rates on the T_1 resistance plants were similar to that on NBB5 and 100-fold lower than that on Nipponbare. In addition, the T_1 plants were also tested with the specific PCR marker for *Xa5*, showing that all the susceptible plants had the transgene while the resistant plants all lacked the transgene (data not shown). Thus, we confirmed that the rice *TFIIA*γ gene functions as *xa5*.

Comparison of the expression patterns among *Xa5*, *xa5*, and *TFIIA*γ1

By using RT-PCR with a pair of primers for *Xa5* and *xa5*, Iyer and McCouch (2004) found that the transcripts of *Xa5* and *xa5* were detectable at all time points in the leaves of both resistant and susceptible plants with and without inoculation of *Xoo* race 2. And also they mentioned that *TFIIA*γ1 expressed in adult plants at lower levels than *Xa5* or *xa5* though the detail was not shown. To further compare the expression patterns between *TFIIA*γ5 (*Xa5*/*xa5*) and *TFIIA*γ1, two pairs of primers were designed for *Xa5*/*xa5* and *TFIIA*γ1, respectively, and by using RT-PCR for detection of their respective expressions in Nipponbare and NBB5 plants after *Xoo* race P1 or mock inoculation, respectively. The result demonstrated that the *Xa5*, *xa5*, and *TFIIA*γ1 genes are all expressed constitutively during the tested time course in leaves at similar levels (Fig. 4a–d). In addition, we examined the respective expression of *Xa5*/*xa5* and *TFIIA*γ1 in other rice organs including stem, root, and panicle. Interestingly, in panicle, constitutive expression could only be detected for *Xa5* (*xa5*) but not for *TFIIA*γ1. Both genes were expressed in stem and root at similar levels (Fig. 4e).

Comparison of the two *TFIIA*γ-like genes and their proteins in rice

Sequence comparison of the *Xa5* and *TFIIA*γ1 gene revealed 78.8 and 85.8% identity at the nucleotide and amino acid level, respectively. Both share high degrees of

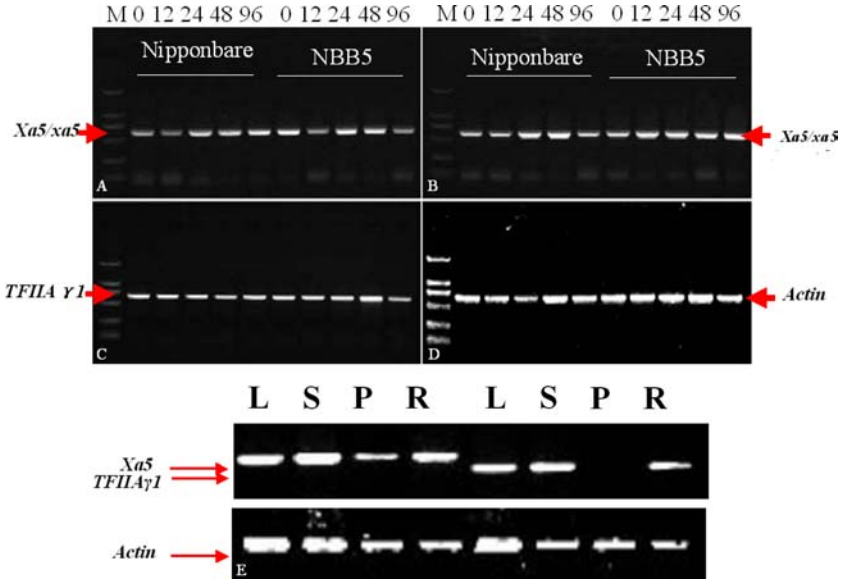

Fig. 4 *TFIIAγ*1 has an expression pattern similar to *Xa5* and *xa5*. **a** Expression of *Xa5*/*xa5* gene in rice leaves upon inoculation with Philippine race 1. **b** Expression of *Xa5*/*xa5* gene in rice leaves upon inoculation with water mock. **c** Expression of *TFIIAγ*1 gene in rice leaves upon inoculation with Philippine race 1. **d** Expression of actin gene in rice leaves upon inoculation with Philippine race 1. **e** Expression of *Xa5*/*xa5* and *TFIIAγ*1 gene in different organs of rice plant (*M* = DL2000; the *number* shows hours; *arrow* shows the targeted bands; *L*, *S*, *P*, and *R* indicate leaf, stem, panicle, and root, respectively)

nucleotide and amino acid sequence similarity with the Arabidopsis TFIIA small subunit gene (*atTFIIAγ*), and contain two conserved domains found in all small subunit of TFIIA, i.e., four helix-bundle domains in N-termini and 12*β*-sheet domains in the C-termini (Geiger et al. 1996; Tan et al. 1996). As shown in Fig. 5, *Xa5* and *TFIIAγ*1 share 70 and 66.1% identity with the *atTFIIAγ* gene of *Arabidopsis*, respectively. By aligning Xa5 and TFIIA*γ*1 with other TFIIA small subunit proteins in other eukaryotes (Fig. 6a), we can see that the Xa5 protein is highly related to the small subunit of TFIIA from human, baker's yeast, fission yeast, fruit fly,

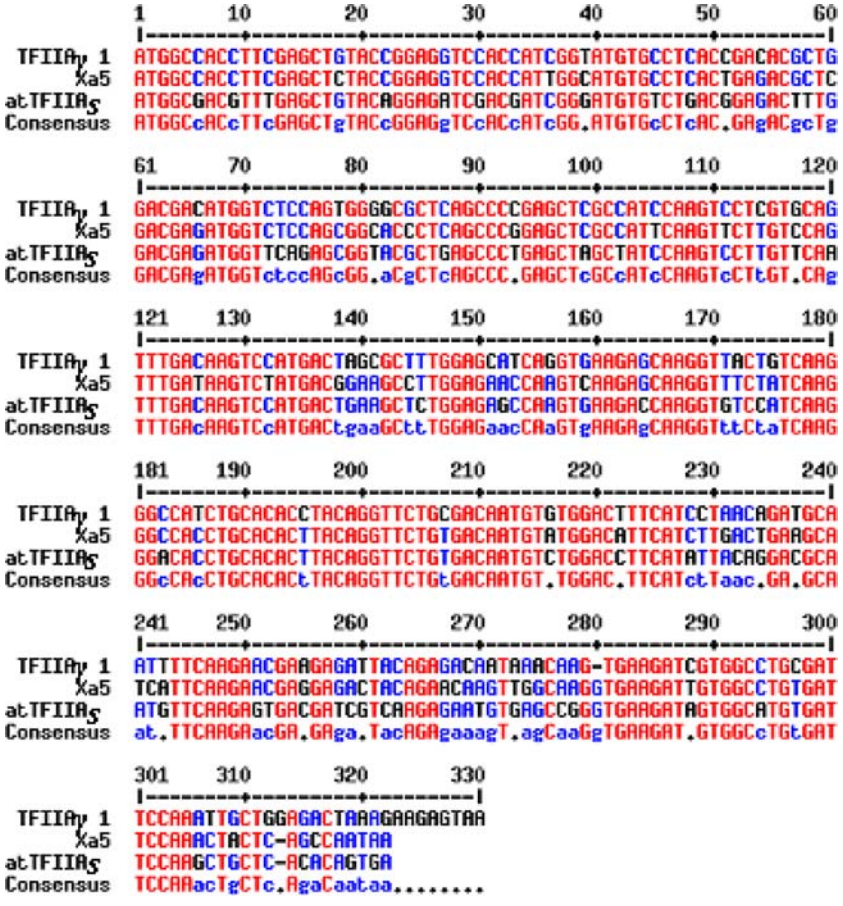

Fig. 5 Alignment of *Xa5*, *TFIIAγ*1 and the gene for the small subunit basic transcription factor TFIIA of *Arabidopsis* (*atTFIIAs*)

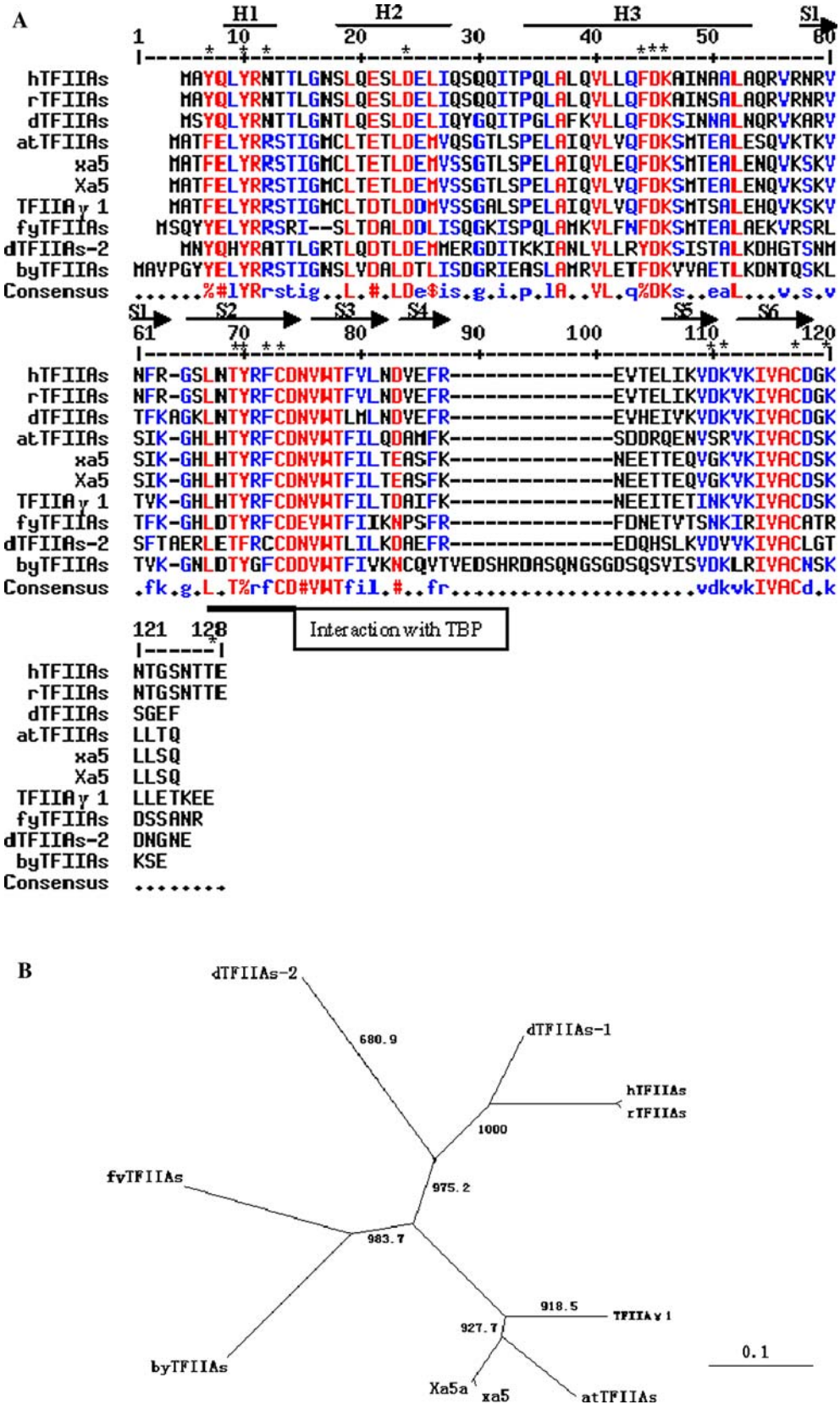

Fig. 6 Amino acid sequence alignment and phylogenetic analyses of Xa5, xa5, TFIIAγ1 and the TFIIA small subunit from other eukaryotes. **a** Amino acid sequence alignment of Xa5, TFIIAγ1, and other TFIIA small subunits (http://www.prodes.toulouse.inra.fr/multalin/). There are two conserved domains in the N-terminal and C-terminal of the xa5 protein. Mutations that affect human TFIIAs transcriptional activity are indicated by a *diamond* (Ozer et al. 1996). Sequences of yTFIIAs that interact with TBP and DNA are marked by *dark bar* (Tan et al. 1996). H indicates α-helices, and S indicates β-strands. GenBank accession numbers: Fission Yeast yTFIIAs (O74948), Arabidopsis atTFIIAs (Q39236), Rat rTFIIAs (O08950), Fruit fly dTFIIAs-1 (P52656) and dTFIIAs-2 (Q9W5B9), Human hTFIIAs (XP_510452), Baker's yeast byTFIIAγs (NP_012865). Identical residues are marked in *red*, similar ones in *blue*. *Dashes* are gaps introduced to maximize alignment. Mutations that disrupt TFIIA small subunit to interact with TBP or DNA, even affect its transcriptional activity in human, are indicated by a *dark prism* (Ozer et al. 1996). **b** The unrooted phylogenetic tree was constructed by the neighbor-joining method using PHYLIP (http://www.bioweb.pasteur.fr/). The numbers above the branches represent the bootstrap support in 1,000 replicates. The scale bar is an indicator of genetic distance based on branch length

rat, and Arabidopsis (47.2, 41.5, 48.1, 35.8–45.3, 47.2, and 85.8% identity, respectively). To determine the evolutionary relationships of Xa5/xa5, TFIIAγ1, and other TFIIAγ, a phylogenetic tree for these protein sequences was built by using the neighbor-joining method (Saitou and Nei 1987). The final unrooted tree indicates that Xa5/xa5 is closer to the TFIIA small subunit of *Arabidopsis* than TFIIAγ1, though they all grouped into the same family (Fig. 6b).

Prediction of 3-D structures of Xa5 and TFIIAγ1 proteins

The crystal structures of yeast and Human TFIIA small subunit were solved by X-ray crystallography (Tan et al. 1996; Geiger et al. 1996; Bleichenbacher et al. 2003). By comparing the rice Xa5, xa5, and TFIIAγ1 proteins with the TFIIA small subunits that have been analyzed in structure, we would learn which one of them is more likely to function as a typical TFIIA small. To address the question, 3-D structures of the three proteins were predicted by homology modeling using computer-aided modeling techniques. As Xa5, xa5, and TFIIAγ1 are highly homologous to the small subunits of human, yeast, and *Drosophila* TFIIAγ (Bernstein et al. 1994; DeJong et al. 1995; Ozer et al. 1996; Tan et al. 1996), the D chain of human TFIIA (PDB ID: 1NVP) was selected as the initial homology template. After the crude initial structures of Xa5, xa5, and TFIIAγ1 were predicted by knowledge-based homology modeling, they were optimized by a combination of energy minimization and molecular dynamics (Fig. 7a). In addition, to determine whether the resistance function of xa5 is related to the structure of its active site, we superimposed backbones of the four structures. Notably, these structures superimpose very well in most of the regions, only the glutamic acid that substitutes valine at the 39th position in the xa5 protein leads to a small structural change in its third helix domain as shown in Fig. 7b–d. The root mean square deviations (RMSDs) of Xa5 and xa5 from the D chain of human TFIIA are 0.405356 and

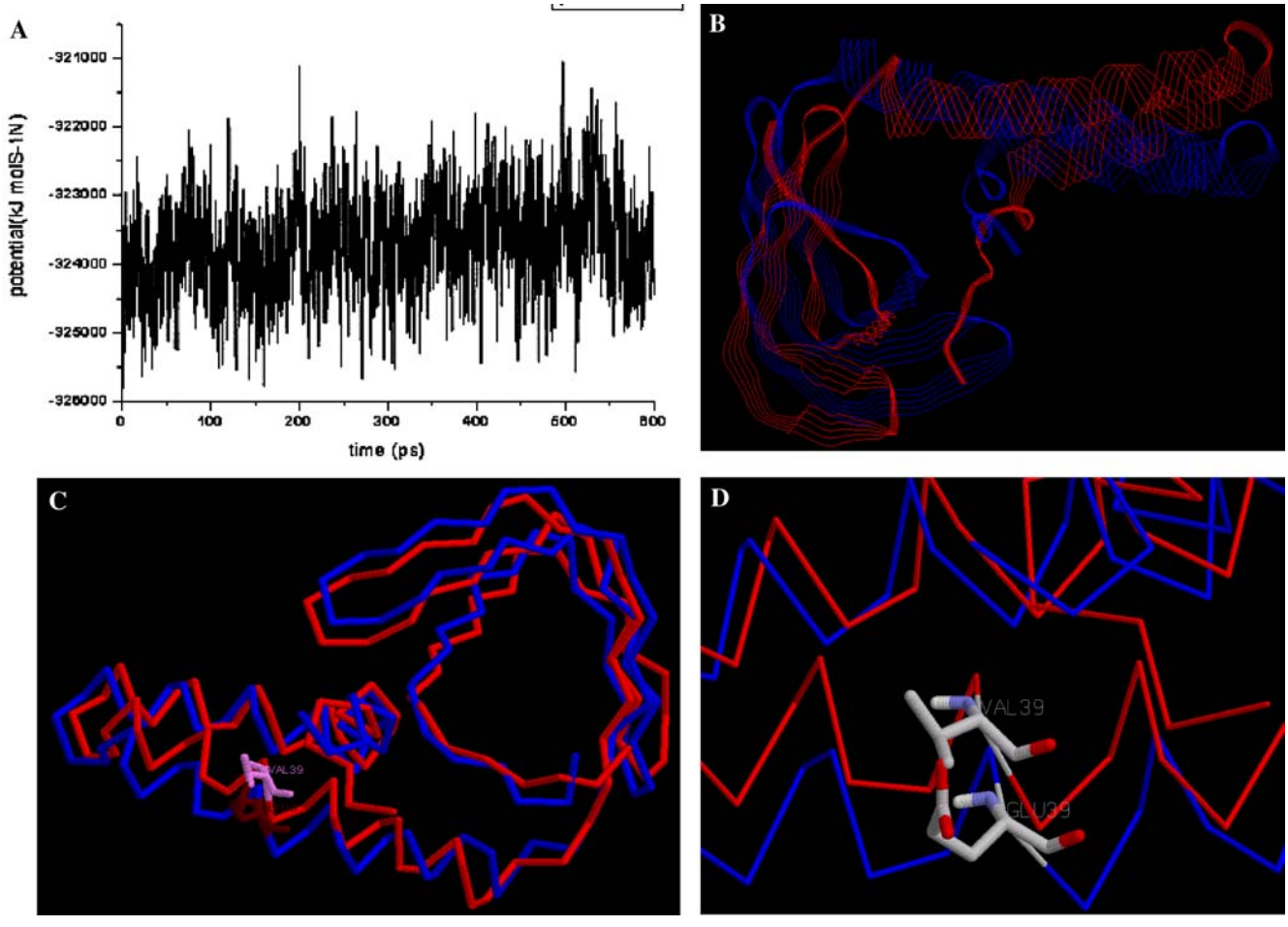

Fig. 7 Prediction of 3-D structures of Xa5, xa5, and TFIIAγ1 proteins. **a** Potential–time curve of last 600 ps. **b** Superimposed structure of the predicted model of Xa5 and the X-ray structure starting scaffold (1VIP, *blue ribbon*), their RMSD is 0.405356 Å. **c** Superimposed structure of the predicted model of Xa5 (*red backbone*) and xa5, their RMSD is 0.239546 Å. **d** The enlarged substituted site in the predicted xa5 model, the *red backbone* is Xa5

0.39655 Å, respectively, while the RMSDs of TFIIAγ1 and xa5 from Xa5 are 0.559522 and 0.239546 Å, respectively. This small variation between xa5 and Xa5 may implicate the functional difference between *xa5* and *Xa5*.

Discussion

In most complementation tests, the phenotype of a dominant transgene can be viewed and evaluated clearly in the T_0 transgenic plants, while the recessive phenotype can only be distinguished in self-pollinated progenies of the T_0 plants. In this study, we chose to transfer the dominant *Xa5* with the associated trait of disease susceptibility to complement the resistance phenotype of *xa5*, so that the transformed phenotype could be detected in T_0 plants directly. At the same time, the *indica* rice like IRBB5 is very difficult to be transformed and we chose to generate an easily transformed line homozygous for *xa5* genes in a genetic background similar to Nipponbare (*japonica* rice) by crossing IRBB5 with Nipponbare. With this line, we successfully complemented *xa5* with *Xa5* gene. This approach should be applied to other cases where mutant genes have been identified from *indica* rice lines that are difficult to transform, and the function of the wild allele should be confirmed by complementation test.

Through the above-mentioned approach, the TFIIA-like gene with the V39E variation on chromosome 5 was functionally confirmed as the rice *Xoo* resistance gene *xa5*. Notably, rice has two TFIIA-like genes, and the other gene, *TFIIAγ1*, on chromosome 1 has no relationship to the disease resistance or susceptibility. Several lines of evidences suggest that the two genes, *Xa5*/*xa5* and *TFIIAγ1*, did not diverge too much from each other in the evolution of rice to lose the basic function of their encoded small subunits of the rice TFIIA. Firstly, the two genes were found both constitutively expressed in different rice organs with the exception in panicle where the expression of *TFIIAγ1* could not be detected by RT-PCR (Fig. 4; the data by Iyer and McCouch 2004), Secondly, the deduced Xa5/xa5 and TFIIAγ1 protein sequences show the striking sequence characteristics in the α-helix bundle and β-sheet domain that are conserved across yeast, *Drosophila*, human, rat, and *Arabidopsis*, and all the three contain most of the amino acid residues that were considered essential to the TFIIA small subunit's activity (Ozer et al. 1996; Fig. 6a). Thirdly, the 3-D structures of Xa5/xa5 and TFIIAγ1 superimposed very well with the X-ray structure of TFIIA small subunit from human. Besides, it is also worth mentioning that the substitution of V39E occurs in the α-helix domain of the xa5 protein, a supposed conservative substitutable site. This amino acid variation may lead to a small change in the third helix domain of the xa5 protein, but it should not affect the basal transcription function of TFIIA.

Based on the above considerations, *xa5* must confer its resistance to bacterial blight via a novel pathway. Iyer and McCouch (2004) provided two possible models for the *xa5* resistance. One is that the xa5 protein interaction with the acidic transcription activation domains (AAD), the domain of Avrxa5 to retard host cell transcription and results in rapid cell death and resistance; the other is that the interaction between xa5 and Avrxa5 could be recognized by a certain nuclear protein to initiate defense responses. Here we propose another model.

It has been known that TFIIA can directly stimulate the TATA binding protein (TBP) binding to the TATA box (Buratowski et al. 1989; Maldonado et al. 1990; Lee et al. 1991; Imbalzano et al. 1994) while, in this process, it competes with repressor for binding to TBP and acts as a derepressor to prevent non-productive preinitiation complex formation (Meisterernst and Roeder 1991; Roeder 1991; Inostroza et al. 1992; Cortes et al. 1992; Auble and Hahn 1993; Auble et al. 1994; Merino et al. 1993). In addition, TFIIA is required for transcriptional activation regulated by some specific transcription factors (DeJong et al. 1995; Orphanides et al. 1996; Ozer et al. 1996; Liu et al. 1999, Li et al. 1999). Recently, a transcription activation domain was revealed in the TFIIA large subunit of *Arabidopsis* (Li et al. 1999), and the transcription factor GT-1 that specifically binds to Box II, a promoter *cis*-element with the core DNA sequence 5′-GGTTAA in many defense-related genes such as CHS and PR-1, was shown to interact with TFIIA–TBP–TATA complex via TFIIA (Gourrierec et al. 1999). Interestingly, the Box II-related sequences may have either a positive or negative role in the transcription of different genes in different organs. For example, GT-1 was shown to function as a silencer to interact with the Box II element of bean's CHS promoter, and the GT-1 binding activity with the tobacco PR-1a promoter was reduced after challenging with salicylic acid or tobacco mosaic virus (Dron et al. 1988; Lawton et al. 1991; Villain et al. 1994, 1996; Buchel et al. 1996; Lopes Cardoso et al. 1997). On the other side, it is also known that the R-gene products can interact with pathogen elicitors either directly or indirectly (Tang et al. 1996; Baker et al. 1997; Jia et al. 2000; Jones 2001; Nimchuk et al. 2001). Notably, the aviruence (*avrxa5*) gene corresponding to *xa5* is a member of the AvrBs3 protein family whose products can be secreted into the host cells from the bacteria to trigger the hypersensitive response (HR; Hopkins et al. 1992; Rossier et al. 1999). The HR induction by the ArvBs3, AvrXa10, and AvrXa7 proteins is dependent on their nuclear localization signals (Zhu et al. 1998, 1999; Van den Ackerveken et al. 1996; Yang et al. 2000), and their AAD that could interact with TFIIA small subunit as suggested by a coimmuno-precipitation assay (Kobayashi et al. 1995). In addition, some AvrBs3 family proteins have been shown to bind to double-stranded DNA (Yang et al. 2000) and specifically up-regulates genes during infection and in the resistance reaction mediated by Xa27 (Marois et al. 2002; Gu et al. 2005). Based on these observations,

we speculate that the xa5 protein has stronger binding activity to the positive transcription activators such as Arvxa5 than the repressors like GT-1, which may lead to an increase of the TBP–DNA complex and initiates the transcriptions of some specific genes. By contrast, the valine (V) at the 39th position of Xa5 might compromise Xa5's binding to the negative transcription activators and, therefore, Xa5 would have a comparatively stronger binding activity to the negative regulator like GT-1 and work as a silencer to some defense-related genes. With this model in mind, we think that the observed intermediate, but variable, lesion lengths in the *Xoo*-inoculated F_1 individuals (*XA5xa5*) from the IR24×IRBB5 cross by Li et al. (2001) and Iyer and McCouch (2004) may be the results of the competitions between xa5-activators and Xa5-repressors in the heterozygous plants.

The four cloned dominant *Xoo*-R-genes, *Xa21* (Song et al. 1995), *Xa1* (Yoshimura et al. 1998), *Xa26* (Sun et al. 2004), and *Xa27* (Gu et al. 2005), have been shown to encode three different types of R-proteins, respectively, indicating that the rice resistance to bacterial blight could be a complicated process which may involve different pathways. Further characterization of the function of *xa5* will deepen our understanding into a unique signal transduction pathway that leads to formation of a recessive resistance. In the present study, by comparing the expression patterns between *Xa5*/*xa5* and *TFIIA*γ1 and the 3-D structures between Xa5/xa5 and TFIIAγ1, we could come to a conclusion that *Xa5*/*xa5* seems have not been specialized yet as a resistance gene only and still keep its function as a basic transcription factor in the evolutional process since the Xa5/xa5 and TFIIAγ1 diverged from each other. Biochemical and molecular testing their respective functions will help us to elucidate how a gene with a basic function could gain a new function for disease resistance by gene duplication in evolution. In addition, such a study will clarify the true phylogenic relationship between the TFIIA small subunit gene in Arabidopsis and its homologous copies in rice.

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